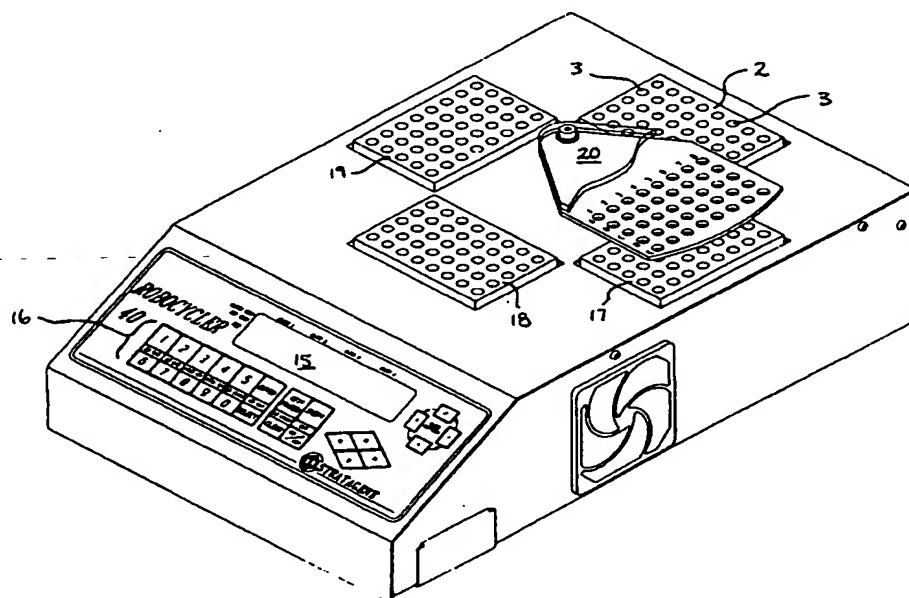




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12M 1/38	A1	(11) International Publication Number: WO 95/11294 (43) International Publication Date: 27 April 1995 (27.04.95)
(21) International Application Number: PCT/US94/12023 (22) International Filing Date: 20 October 1994 (20.10.94) (30) Priority Data: 139,540 20 October 1993 (20.10.93) US (71) Applicant (for all designated States except US): STRATAGENE [US/US]; 11099 North Torrey Pines Road, La Jolla, CA 92037 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): DANSSAERT, John, L. [US/US]; 9735 Caminito Doha, San Diego, CA 91231 (US). SHOPES, Robert, J. [US/US]; 1639 28th Street, San Diego, CA 92102 (US). SHOEMAKER, Daniel, D. [US/US]; Abrahms Apartment 7C, Escondido Village, Stanford, CA 94305 (US). (74) Agents: HALLUIN, Albert, P. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>

(54) Title: THERMAL CYCLER INCLUDING A TEMPERATURE GRADIENT BLOCK



(57) Abstract

A method in which a temperature is generated across a gradient block, and an apparatus comprising a block (2, 17, 18, 19) across which a temperature gradient can be generated. By setting up such a gradient, multiple reaction mixtures held in wells (3) on the gradient block (2, 17, 18, 19) can be simultaneously run at temperatures which differ only slightly, thereby permitting an optimum temperature for the reaction to be quickly identified. In a preferred embodiment, the gradient block (2, 17, 18, 19) is integrated into a thermal cycler used for nucleic acid amplification reactions.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	ML	Mali	TJ	Tajikistan
DE	Germany	MN	Mongolia	TT	Trinidad and Tobago
DK	Denmark			UA	Ukraine
ES	Spain			US	United States of America
FI	Finland			UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

THERMAL CYCLER INCLUDING A TEMPERATURE GRADIENT BLOCKFIELD OF THE INVENTION

The present invention relates to a temperature cycling
5 apparatus useful for performing nucleic acid amplification,
DNA sequencing and the like which apparatus can include single
or multiple heating and/or cooling blocks containing sample
wells wherein a temperature gradient can be generated across a
given block.

10

BACKGROUND OF THE INVENTION

Systems which require multiple or cyclic chemical
reactions to produce a desired product often require careful
temperature control to produce optimal results. Such
15 reactions include nucleic acid amplification reactions such as
the polymerase chain reaction (PCR) and the ligase chain
reaction (LCR). For this reason, apparatus have been
developed which permit the accurate control of the temperature
of reaction vessels in which such amplification reactions are
20 performed.

For example, there are a number of thermal "cyclers" used
for DNA amplification and sequencing in the prior art in which
one or more temperature controlled elements or "blocks" hold
the reaction mixture, and the temperature of a block is varied
25 over time.

Another prior art system is represented by a temperature
cycler in which multiple temperature controlled blocks are
kept at different desired temperatures and a robotic arm is
utilized to move reaction mixtures from block to block.

30 All of these systems include features which allow the
user to program temperatures or temperature profiles over time
for a block on the instrument so that various processes (e.g.
denaturing, annealing and extension) can be efficiently
accomplished once the optimum temperatures for these steps are
35 determined. Importantly, however, the determination of the
optimum temperature for each of the various steps in any
reaction system, and in particular for any nucleic

amplification or incubation reaction involving an annealing step, is not a simple task.

PCR is a technique involving multiple cycles that results in the geometric amplification of certain polynucleotide sequence each time a cycle is completed. The technique of PCR is well known to the person of average skill in the art of molecular biology. The technique of PCR is described in many books, including, PCR: A Practical Approach, M.J. McPherson, et al., IRL Press (1991), PCR Protocols: A Guide to Methods and Applications, by Innis, et al., Academic Press (1990), and PCR Technology: Principals and Applications for DNA Amplification, H.A. Erlich, Stockton Press (1989). PCR is also described in many U.S. patents, including U.S. patents, 4,683,195; 4,683,202; 4,800,159; 4,965,188; 4,889,818; 5,075,216; 5,079,352; 5,104,792; 5,023,171; 5,091,310; and 5,066,584, which are hereby incorporated by reference.

The PCR technique typically involves the step of denaturing a polynucleotide, followed by the step of annealing at least a pair of primer oligonucleotides to the denatured polynucleotide, i.e., hybridizing the primer to the denatured polynucleotide template. After the annealing step, an enzyme with polymerase activity catalyzes synthesis of a new polynucleotide strand that incorporates the primer oligonucleotide and uses the original denatured polynucleotide as a synthesis template. This series of steps (denaturation, primer annealing, and primer extension) constitutes a PCR cycle. As cycles are repeated, the amount of newly synthesized polynucleotide increases geometrically because the newly synthesized polynucleotides from an earlier cycle can serve as templates for synthesis in subsequent cycles. Primer oligonucleotides are typically selected in pairs that can anneal to opposite strands of a given double-stranded polynucleotide sequence so that the region between the two annealing sites is amplified.

The temperature of the reaction mixture must be varied during a PCR cycle, and consequently varied many times during a multicycle PCR experiment. For example, denaturation of DNA

typically takes place at around 90-95°C, annealing a primer to the denatured DNA is typically performed at around 40-60°C, and the step of extending the annealed primers with a polymerase is typically performed at around 70-75°C. Each of these steps has an optimal temperature for obtaining the desired result. Many experiments are required to determine the optimal temperature for each step.

For example, while the temperature at which DNA denatures is generally between 90-95°C, slight variations in the particular temperature necessary are observed depending on the length of the DNA and the percentage of each of the four deoxynucleotides present (guanine-cytosine pairs and adenine-thymine pairs). Insufficient heating during the denaturation step is a common reason for a PCR reaction to fail. However, overheating during the denaturation step can result in excessive denaturation of the polymerase.

Achieving the optimal temperature for the PCR annealing step is even more critical. An annealing temperature which is too low will result in non-specific DNA fragments being amplified. At too high of an annealing temperature, the primers will anneal less efficiently resulting in decreased yield of the desired product and possibly reduced purity. In the annealing step, the optimal temperature will depend on many factors including the length of the primer and the percentage of each of the four deoxynucleotides present (guanine-cytosine pairs and adenine-thymine pairs). For a typical 20-base oligonucleotide primer comprised of roughly 50% guanine-cytosine, a temperature of 55°C is a good estimate for the lower end of the temperature range. However, as one increases the primer length in order to attain greater primer specificity, differing annealing temperatures may be required. Thus, the number of subtle influences on the optimal annealing temperature makes difficult the task of quickly identifying the optimum for a given system.

Achieving the optimal temperature for the extension reaction is also important for obtaining the desired PCR result. Temperature may affect both the rate and the accuracy

of the extension reaction. If the rate of the polymerase reaction is too low, then the newly synthesized polynucleotide may not contain a site for primer annealing. Additionally, the denatured polynucleotide sequence for amplification may contain one or more regions of secondary structure that may form or disappear according to the temperature selected. Furthermore, several different enzymes with polymerase activity may be used for PCR. Each enzyme will have its own optimum temperature for activity, stability and accuracy.

10 Determination of the optimal denaturing, annealing, and extension temperatures for a particular PCR is complicated by the fact that the optimum will be different for each of the reactions. Thus, in order to determine the three optimal temperature ranges, multiple separate experiments must be run
15 where two temperature variables are held constant while a third temperature variable is changed. As a result, determination of the optimal temperature for running a PCR system can be a time consuming task.

It is therefore an object of the present invention to
20 provide an efficient means by which optimal reaction temperatures can be more efficiently identified for PCR and other reactions.

SUMMARY OF THE INVENTION

25 To achieve this object, the invention is a method in which a temperature gradient is generated across a "gradient" block. The invention also includes an apparatus comprising a block across which a temperature gradient can be generated. By setting up such a gradient, multiple reaction mixtures can
30 be simultaneously run at temperatures which differ only slightly, thereby permitting an optimum temperature for a given reaction to be quickly identified. In the most preferred embodiment of the invention the gradient block is integrated into a thermal cycler. By doing so, it is possible
35 to run a series of desired reactions using the thermal cycler immediately upon identification of the optimum reaction temperature.

In a first embodiment, the invention is a method for simultaneously reacting a plurality of reaction mixtures in an apparatus including a temperature gradient block comprising the steps of:

- 5 placing reaction mixtures in a plurality of reaction wells in the gradient block, the gradient block having a top portion, first and second opposing portions, and a bottom portion, the plurality of reaction mixture wells being formed in the block between the opposing portions, and
- 10 generating a temperature gradient across said gradient block and between the opposing portions.

In this embodiment, the step of generating a temperature gradient may comprise the steps of heating the first opposing portion of the gradient block, and cooling the second opposing
15 portion of the gradient block. The method may also include the step of controlling the temperature gradient using a controlling means. By using a controlling means, the method may further include the steps of collecting and storing temperature set point and actual temperature data from the
20 wells, and transmitting that information to a microprocessor.

In another form of the method of the invention, where the apparatus further comprises at least one additional heat conducting block having a top portion, first and second opposing portions, and a bottom portion, and a plurality of
25 reaction mixture wells formed in the additional block between the opposing portions, the method may further comprise the step of moving the reaction mixtures between the gradient block and one or more of the additional block or blocks.

In another form, the method employs an apparatus
30 comprising at least one heat conducting block, the block having a plurality of sample wells spaced between first and second opposing portions and in an upper surface thereof, and the method comprises

- 35 placing reaction mixtures in the wells, and
- generating a temperature gradient across the block and between the opposing portions by heating the first opposing portion and cooling the second opposing portion.

The invention also includes an apparatus comprising:

a reaction mixture holder, the reaction mixture holder comprising a heat conducting block having a top portion, first and second opposing portions, and a bottom portion, a
5 plurality of reaction mixture wells formed in the top portion, and between the first and second opposing portions,

a block heater positioned adjacent to the first opposing portion, and

a block cooler positioned adjacent to the second opposing
10 portion.

In another form, the apparatus of the invention comprises holding means for holding a reaction mixture, the holding means including a heat conducting block having a top portion, first and second opposing portions, and a bottom portion, and
15 a plurality of reaction mixture wells formed in the top portion and between the first and second opposing portions; and means for generating a temperature gradient across the heat conducting block and between the first and second opposing portions.

20 In yet another form, the invention includes an apparatus for performing molecular biological reactions comprising at least one temperature controlled block, the block having a plurality of reaction mixture wells spaced between first and second opposing portions and in an upper portion thereof, and
25 a block heater positioned adjacent to the first opposing portion and capable of generating a temperature gradient between the first and second opposing portions.

In a preferred embodiment, the heat conducting block or "gradient" block is made substantially of, or comprises,
30 brass.

The apparatus of the invention can include additional elements. Thus, in an especially preferred embodiment, the apparatus includes more than one heat conducting block along with the gradient block. The apparatus may also include a
35 controller for controlling the temperature gradient across the gradient block, and in a multi-block apparatus, the controller may also control the temperature of blocks which are heated or

cooled to a uniform temperature. Preferably, the controller will include a microprocessor for collecting and storing temperature set point and actual temperature data, and multiple temperature sensors for collecting the actual
5 temperature data from the wells and for transmitting the information to the microprocessor.

In another embodiment, the plurality of wells in the gradient block are formed in parallel, aligned rows. Further, where more than one block is included, the apparatus may
10 include a robot arm for moving samples between blocks in a programmably controllable manner.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood by reference to
15 the appended figures of which:

Figure 1 is a perspective view of a thermal cyclor incorporating the thermal gradient block of the invention;

Figure 2 is a perspective, exploded view, of the thermal gradient block, surrounding heaters and cooler according to
20 the invention; and

Figure 3 is a block diagram depicting the elements of a thermal cyclor in which the thermal gradient apparatus and method of the invention may be used.

25 DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a device and method for creating a thermal gradient across a block, such as a block in known thermal cyclers for PCR reactions, which enables one to simultaneously conduct a series of experiments at very close
30 to the same temperatures. As used herein, the term "block" refers to a structure, usually metal, which can be temperature controlled and in which wells have been arranged to accept vessels containing reaction mixtures or "samples." The phrase "gradient block" as used herein is intended to describe such a
35 block, except that a gradient block is a block across which a temperature gradient can be established. Examples of the specific manner in which such a temperature gradient can be

established are discussed herein, though those skilled in the art will understand that once the advantage of having a gradient block is known, many other variations of the apparatus shown herein can be easily identified.

5 One particular area of utility for the present invention is in multiple block thermal cyclers. By incorporating the gradient block of the invention into a multiple block thermal cycler, it is possible to simultaneously conduct a series of reactions where the temperature at which the reactions are
10 proceeding is varied across the gradient block. This permits the rapid determination of the optimal temperature for that particular reaction.

Figure 1 depicts the prior art Stratagene device in which a thermal gradient block 2 according to the invention has been
15 incorporated.

Various components of the cycler depicted in greater detail in Figures 2 and 3 can be seen in Figure 1, i.e. display 15, keypad 16, additional blocks 17, 18 and 19 and robot arm 20 (shown in cut-away view).

20 It will be understood that a microprocessor can be incorporated into the control electronics of the apparatus, as is well known. The microprocessor can be used to control the range of the temperature gradient and also to program the movement of samples into and out of the thermal gradient
25 block. The microprocessor executes commands written in software that collect user input via the keyboard, compare the input to actual temperatures, and turn off or on the heating or cooling units as appropriate. The electronics also
30 the microprocessor to compare the elapsed time that the reaction mixture has been in a given block and compare it to a desired time input by the user.

The microprocessor will also control the robot arm, which arm is operated using two stepper motors. One motor raises
35 and lowers the arm. The other rotates the arm from block to block.

Thus, those skilled in the art can readily understand how the thermal gradient block of the invention can be incorporated into known thermal cyclers.

Of course, the thermal gradient block of the invention need not necessarily be incorporated into a known cycler to be advantageously used. For example, a stand alone unit incorporating the thermal gradient block could be used in conjunction with known cyclers so that optimum reaction temperatures could be identified and then used in those cyclers.

Figure 2 provides an exploded view of the components of the gradient block assembly. Thus, in Figure 2, the gradient block apparatus is generally designated by reference number 1. The apparatus comprises a heat conducting block 2 which incorporates a number of wells 3 for holding reaction mixtures or the vessels in which the mixtures can be held. In a portion of block 2, heater 5 fits into opening 4. Heater 5 is a commonly available cylindrically shaped cartridge type resistive heater (RAMA brand, San Jacinto, California).

Depending on the temperature range desired, the opposing portion of block 2 may simultaneously be cooled using a heat sink made up of a ribbed aluminum block 7 and a fan 9. Naturally, whether the heat sink is operated or not, a temperature gradient will be created between the opposing portions of the block. However, where the temperature gradient is to be made larger, the heat sink can be operated. To enhance the ability to create and maintain a gradient, block 2 is preferably composed of a material with a relatively low coefficient of thermal conductivity to reduce the amount of heat flux necessary to create the temperature gradient across the block. Brass is preferred.

Where a multiblock system is used (Figure 1) blocks other than the gradient block will be constructed of a material with a relatively high coefficient of thermal conductivity. By doing so, the blocks can be heated or cooled to a uniform temperature but will not be thermally conductive enough to

require excess heating or cooling to maintain a temperature. Aluminum is known for such uses in the prior art.

Depending on the size of the gradient block and the heating and cooling capacities of the heater and heat sink, 5 temperature gradients in excess of 1°C to 22°C, preferably 1°C to 14°C, across block 2 can be achieved. Holes 6 may be drilled in block 2 to limit thermal conductivity, such that parallel aligned rows of wells in the block tend to be at one temperature. The use of holes also permits the temperature 10 profile across the gradient block, and from one row of wells to the next, to be linear.

Heaters and coolers known in the art may be used. For example, Peltier thermoelectric devices may be used, though other passive or active heaters would also be useful (e.g. 15 chilled or heated liquids or gases).

As shown in both Figures 1 and 2, gradient block 2 preferably has eight to twelve rows of sample wells 3 equally spaced across the block. Each row can contain five to eight sample wells. 0.2 or 0.5 ml tubes can be used. The 20 particular number and design of the sample wells can be varied to modify capacity if desired. If a temperature gradient of 7°C to 11°C is formed between the hot and cold portions of the block, each row of sample wells will differ in temperature by approximately 1°C.

25 Returning to Figure 2, additional heaters 8 and 10 may also be employed so that the system can be operated in the same manner as blocks known in the art, i.e. with uniform heating across the entire length and width of block 2. Heaters 8 and 10 are preferably thin foil type (MINCO brand 30 Minneapolis, Minnesota). Heaters 8 and 10 can also be in conjunction with heater 5 to bring block 2 to at least the cool portion temperature as quickly as possible when the system is started or the temperature range over which block 2 is to be operated is raised.

35 Wire connectors 11, 12 and 13 connect the heaters to a power source. Apparatus 1 may also include a thermostat 14

which can be used as a high temperature cut-off, which is a desirable safety feature.

The block diagram of Figure 3 depicts a gradient block (labelled "second block") of the type shown in Figure 2 as block 2 integrated into a thermal cycler having multiple heating and cooling blocks. The labels in Figure 3 are self-explanatory, and the apparatus described by Figure 2 differs from a known thermal cycler only with respect to the substitution of the gradient block for a non-gradient block.

For PCR, the first, second and third blocks in Figure 3 may be programmed to be maintained at a temperature range of between about 25 to 99°C, and are used for denaturing, annealing and extension respectively. The fourth block is generally maintained at between 4 and 25°C and is used for sample storage after the PCR reaction has completed. The second block, made of brass, will be used for the annealing step.

As can be seen in Figure 3, more than one thermocouple can be used along the length of the gradient block so that temperatures along the block can be carefully monitored and used to feed information back to the control electronics and display.

The following examples are offered for the purpose of illustrating, not limiting, the subject invention.

EXAMPLE 1

USE OF THE GRADIENT THERMAL CYCLER FOR THE POLYMERASE CHAIN REACTION

High temperature primer extension testing of the thermal gradient system of the invention was carried out using two model primer/template systems. These two systems exhibit significantly variable extension product yields depending upon the annealing temperature used during the extension process. Primer/template set #1 amplifies a 105 bp region of the human Gaucher gene, while set #2 amplifies a 540 bp region of the human fucosidase gene. The thermal gradient system of the invention contains a gradient block that allowed primer extension using an optimal annealing temperature range of 42 to 56°C.

METHODS AND MATERIALS

Primer extension reactions were carried out using the gradient block of the invention. Primer/template test set #1 consisted of a genomic human DNA template and two 22mer oligomers yielding a 105 bp extension product. The sequence of primer A was 5' CCTGAGGGCTCCCAGAGAGTGG 3' (SEQ ID NO:1). The sequence of primer B was 5' GGTTTAGCACGACCACAACAGC 3' (SEQ ID NO:2). Primer/template test set #2 consisted of a genomic human DNA template and two oligomers of 20 and 30 bases respectively yielding a 540 bp extension product. The sequence of primer A was 5' AGTCAGGTATCTTTGACAGT 3' (SEQ ID NO:3). The sequence of primer B was 5' AAGCTTCAGGAAAACAGTGAGCAGCGCCTC 3' (SEQ ID NO:4).

The primer extension reaction mixture consisted of 1X Taq DNA polymerase buffer (10 mM tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, .001% (w/v) gelatin), 250 uM each dNTP, 250 ng each primer and template and 2.5 units Taq DNA polymerase in a 100 µl reaction volume. The reaction mixture was overlaid with 50 µl of nuclease free sterile mineral oil.

The temperature cycling parameters used were as follows:

1 min	94°C	
1 min	42-56°C (gradient block)	
1 min	72°C	
1 min	94°C	
1 min	42-56°C (gradient block)	30 cycles
8 min	72°C	
Storage	4°C	

Eight reaction mixes were tested per primer/template set - one per gradient temperature block slot. Annealing temperatures used were 42, 44, 46, 48, 50, 52, 54 and 56°C (two degree C increments across the gradient block). Reactions were carried out in 500 µl tubes.

RESULTS

Both primer/template sets 1 and 2 yielded obviously varying results depending upon the annealing temperature used in the gradient temperature block. Primer extension products

from set #1 varied from the desired single DNA band of size 105 bp (derived from the extension reaction using a 56°C annealing temperature) to a reaction mix yielding multiply sized extraneous DNA extension products (of approximate size 180, 280 and 800 bp) from a reaction using a 48°C annealing temperature. Primer extension products from set #2 varied from the desired single DNA band of size 540 bp (derived from the extension reaction using a 42°C annealing temperature) to a reaction mix yielding an extraneous DNA extension product of approximately 2000 bp from a reaction using a 56°C annealing temperature.

EXAMPLE 2

USE OF THE GRADIENT THERMAL CYCLER FOR THE LIGASE CHAIN REACTION

15

Ligase chain reaction (LCR) is a recently described DNA amplification technique that can be used to detect trace levels of known nucleic acid sequences. LCR involves a cyclic two step reaction which is performed in a DNA thermal cyclor machine. The first step is a high temperature melting step in which double stranded DNA unwinds to become single stranded. The second step is a cooling step in which two sets of adjacent, complementary oligonucleotides anneal to the single stranded target DNA molecules and are ligated together by a DNA ligase enzyme. The products of ligation from one cycle serve as templates for the ligation reaction of the next cycle. Thus, LCR results in the exponential amplification of ligation products.

MATERIALS AND METHODS

30

The materials used in this experiment were obtained from Stratagene, La Jolla, California. The optimal temperature for the second step of the LCR cycle, in which the oligonucleotides are annealed to the DNA target molecules, was determined empirically by the use of the gradient thermal cyclor of the invention. Two sets of reactions were set up, one with a wild type template to which the oligonucleotides

35

were complementary, and one with a mutant template that differed from the wild type template DNA sequence by one base transition. The DNA templates used in this experiment were plasmid constructs containing the pBluescriptII vector and the 5 lac I gene. The wild-type template contained a normal lac I sequence, and the mutant template contained a C to T transition mutation at site 191 within the insert. The four oligonucleotide probes consisted of two pairs of two oligonucleotides each. The first set, A and B, were adjacent to each other and complementary to one strand of the target DNA. The second set, C and D, were complementary to the first set, and therefore occupied adjacent sites on the second strand of the target DNA. The oligonucleotide probe sequences (5' to 3') were as follows:

15

A: TTGTGCCACG CGGTTGGGAA TGTA (SEQ ID NO:5)

B: AGCAACGACT GTTTGCCCGC CAGTTC (SEQ ID NO:6)

C: TACATTCCCA ACCGCGTGGC ACAAC (SEQ ID NO:7)

D: AACTGGCGGG CAAACAGTCG TTGT (SEQ ID NO:8)

20 Oligonucleotide probes A and D were 5'- phosphorylated during synthesis.

The sequence of the wild type lac I insert, starting at site 161 of the insert, was as follows:

5' CTGAATTACA TTCCAACCG CGTGGCACAA CAACTGGCGG GCAAACAGTC

25 GTTGCTGATT 3' (SEQ ID NO:9)

The mutant sequence differed from the wild type by a C to T transition at site 191.

The LCR experiment was performed as follows: The following ingredients were combined in a sterile 500- μ l of 10XZ Pfu LCR buffer, 15 μ l of sterile dH₂O, 1 μ l (10 ng of each) of oligonucleotide mixture, 1 μ l (100 pg) of either the wild-type or mutant plasmid templates or no template, and 1 μ l (4U) of Pfu DNA ligase enzyme. A 25- μ l overlay of sterile mineral oil was added to the tube. This procedure was repeated so that 35 there were a total of 5 tubes each of either the wild type template reaction mixture or the mutant template reaction mixture. The tubes were placed in the gradient thermal cycler

of the invention in positions 1, 3, 5, 7 and 8, so that at each isothermal column in the machine, there would be a wild type and a mutant template reaction. The machine was programmed to cycle between a high temperature of 92°C and the gradient block, which was varied in temperature between 56°C and 70°C. The machine was programmed to move to the high temperature block for 4 minutes, then the gradient block for 3 minutes, then to move between the high temperature block and the gradient block 25 times, stopping for 1 minute at each block. The ligation chain reaction products were visualized by electrophoresis on a 6% polyacrylamide gel buffered with TBE, followed by staining with ethidium bromide and photography under UV light.

15 RESULTS:

The wild type template reaction produced the most intense positive signal in position 8, which corresponds to the coldest (56°C) section of the gradient block. The use of the gradient thermal cyclers of the invention allowed the empirical determination of the best annealing temperature for this reaction in one experiment.

There are many modifications and variations of the thermal gradient block which can advantageously be incorporated into it or related structures. Further, multiple thermal gradient blocks could be employed as more than one block of a multi-block thermal cycler where samples are automatically moved between the various blocks, thereby allowing for multiple reactions to be operated at multiple temperatures.

The invention has been described in detail with respect to its use with PCR. However, in addition to being exceptionally useful for the determination of the optimal temperature for individual steps in a PCR procedure, the invention is also useful for determining the optimal temperature for numerous other chemical reactions. These other chemical reactions include any non-PCR nucleic acid amplification that employs an annealing step analogous to a

PCR annealing step, such as ligase chain reaction (LCR) and DNA cycle sequencing. Other types of reactions for which the invention will be useful include DNA sequencing, cDNA synthesis using a cycling reaction, coupled amplification
5 sequencing (CAS), rapid amplification of cDNA ends (RACE) and any other incubation reaction in which incubations must be accomplished at multiple temperatures.

10

15

20

25

30

35

GENERAL INFORMATION:

- (i) APPLICANT: Denssaert, John L.
Shopes, Robert J.
Shoemaker, Daniel D.
- (ii) TITLE OF INVENTION: THERMAL CYCLER INCLUDING A TEMPERATURE
GRADIENT BLOCK
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Limbach and Limbach
(B) STREET: 2001 Ferry Building
(C) CITY: San Francisco
(D) STATE: CA
(E) COUNTRY: US
(F) ZIP: 94111
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/139,540
(B) FILING DATE: 20-OCT-1993
(C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bengtsson, Patrick
(B) REGISTRATION NUMBER: 32,456
(C) REFERENCE/DOCKET NUMBER: STRG-20130 USA
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 414-433-4150
(B) TELEFAX: 415-433-8716

INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTAGCAC GACCACAACA GC

22

INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGGTAT CTTTGACAGT

20

INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCACG CGGTTGGGAA TGTA

24

INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACGACT GTTGGCCCGC CAGTTC

26

INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCGGG CAAACAGTCG TTGT

24

INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATTACA TTCCCAACCG CGTGGCACAA CAACTGGCGG GCAAACAGTC GTTGCTGATT

60

CLAIMS

What is claimed is:

1. A method for simultaneously reacting a plurality of reaction mixtures in an apparatus including a temperature
5 gradient block comprising the steps of:
 placing reaction mixtures in a plurality of reaction wells in said gradient block, said gradient block having a top portion, first and second opposing portions, and a bottom portion, said plurality of reaction mixture wells being formed
10 in said block between said opposing portions, and
 generating a temperature gradient across said gradient block and between said opposing portions.
2. A method according to claim 1, wherein said step of
15 generating a temperature gradient comprises the step of heating the first opposing portion of the gradient block.
3. A method according to claim 2, wherein said step of generating a temperature gradient comprises the step of
20 cooling the second opposing portion of the gradient block.
4. A method according to claim 1 including the further step of controlling said temperature gradient using
controlling means.
25
5. A method according to claim 4 wherein said controlling step comprises the steps of comparing the temperature set point and the actual temperature data from
said wells.
30
6. A method according to claim 3 wherein said controlling means comprises a microprocessor.
7. A method according to claim 1 wherein said apparatus
35 further comprises at least one additional heat conducting block having a top portion, first and second opposing portions, and a bottom portion, and a plurality of reaction

mixture wells formed in said additional block between said opposing portions, the method further comprising the step of moving said reaction mixtures between said gradient block and said additional block or blocks.

5

8. A method for automated temperature cycling of a reaction mixture using a thermal cycling apparatus comprising at least one heat conducting block, said block having a plurality of sample wells spaced between first and second
10 opposing portions and in an upper surface thereof, the method comprising

placing reaction mixtures in said wells, and
generating a temperature gradient across said block and
between said opposing portions by heating said first opposing
15 portion.

9. A method according to Claim 8, further comprising the step of cooling the second opposing means.

20 10. An apparatus for generating a temperature gradient across a heat conducting block comprising:

a reaction mixture holder, said reaction mixture holder comprising a heat conducting block having a top portion, first and second opposing portions, and a bottom portion, a
25 plurality of reaction mixture wells formed in said top portion, and between said first and second opposing portions,
a block heater positioned adjacent to said first opposing portion.

30 11. An apparatus according to Claim 10 comprising a block cooler positioned adjacent to said second opposing portion.

12. An apparatus for generating a temperature gradient
35 across a heat conducting block according to claim 10 wherein said apparatus further comprises controller means for controlling said block heater.

13. An apparatus according to Claim 12, comprising a cooling block controlled by the controller means.

14. An apparatus for generating a temperature gradient
5 across a heat conducting block according to claim 10 wherein said heat conducting block comprises brass.

15. An apparatus for generating a temperature gradient across a heat conducting block according to claim 12 wherein
10 said controller means comprises a microprocessor for comparing temperature set point and actual temperature data.

16. An apparatus for generating a temperature gradient across a heat conducting block according to claim 10 wherein
15 said plurality of wells in said heat conducting block are spaced across said top portion.

17. An apparatus for generating a temperature gradient across a heat conducting block according to claim 16 wherein
20 said plurality of wells in said heat conducting block are spaced across said top portion in parallel, aligned rows.

18. An apparatus for generating a temperature gradient across a heat conducting block according to claim 10 wherein
25 said holder further comprises a heater positioned adjacent to said bottom portion.

19. An apparatus for generating a temperature gradient across a heat conducting block comprising holding means for
30 holding a reaction mixture, said holding means comprising:
(i) a heat conducting block having a top portion, first and second opposing portions, and a bottom portion, and a plurality of reaction mixture wells formed in said top portion and between said first
35 and second opposing portions; and

(ii) means for generating a temperature gradient across said heat conducting block and between said first and second opposing portions.

- 5 20. An apparatus for generating a temperature gradient across a heat conducting block according to claim 19 wherein said means for generating a temperature gradient comprises means for heating said first opposing portion of said block.
- 10 21. An apparatus according to claim 20 comprising means for cooling said second opposing portion of said block.
22. An apparatus for generating a temperature gradient across a heat conducting block according to claim 19 wherein
15 said apparatus further comprises at least one additional holding means for holding a reaction mixture, said additional holding means comprising:
- 20 (i) at least one additional heat conducting block including a plurality of reaction mixture wells; and
- (ii) means for heating said additional heat conducting block.
23. An apparatus for generating a temperature gradient
25 across a heat conducting block according to claim 19 wherein said heat conducting block comprises brass.
24. An apparatus for generating a temperature gradient across a heat conducting block according to claim 19 wherein
30 said plurality of wells in said heat conducting block are spaced across said block in parallel, aligned rows.
25. An apparatus for generating a temperature gradient across a heat conducting block according to claim 19 wherein
35 said apparatus further comprises controller means for generating said temperature gradient.

26. An apparatus for generating a temperature gradient across a heat conducting block according to claim 25 wherein said controller means comprises a microprocessor for comparing temperature set point and actual temperature data.

5

27. An apparatus for generating a temperature gradient across a heat conducting block according to claim 19 wherein said holder means further comprises a heating means for heating said bottom portion.

10

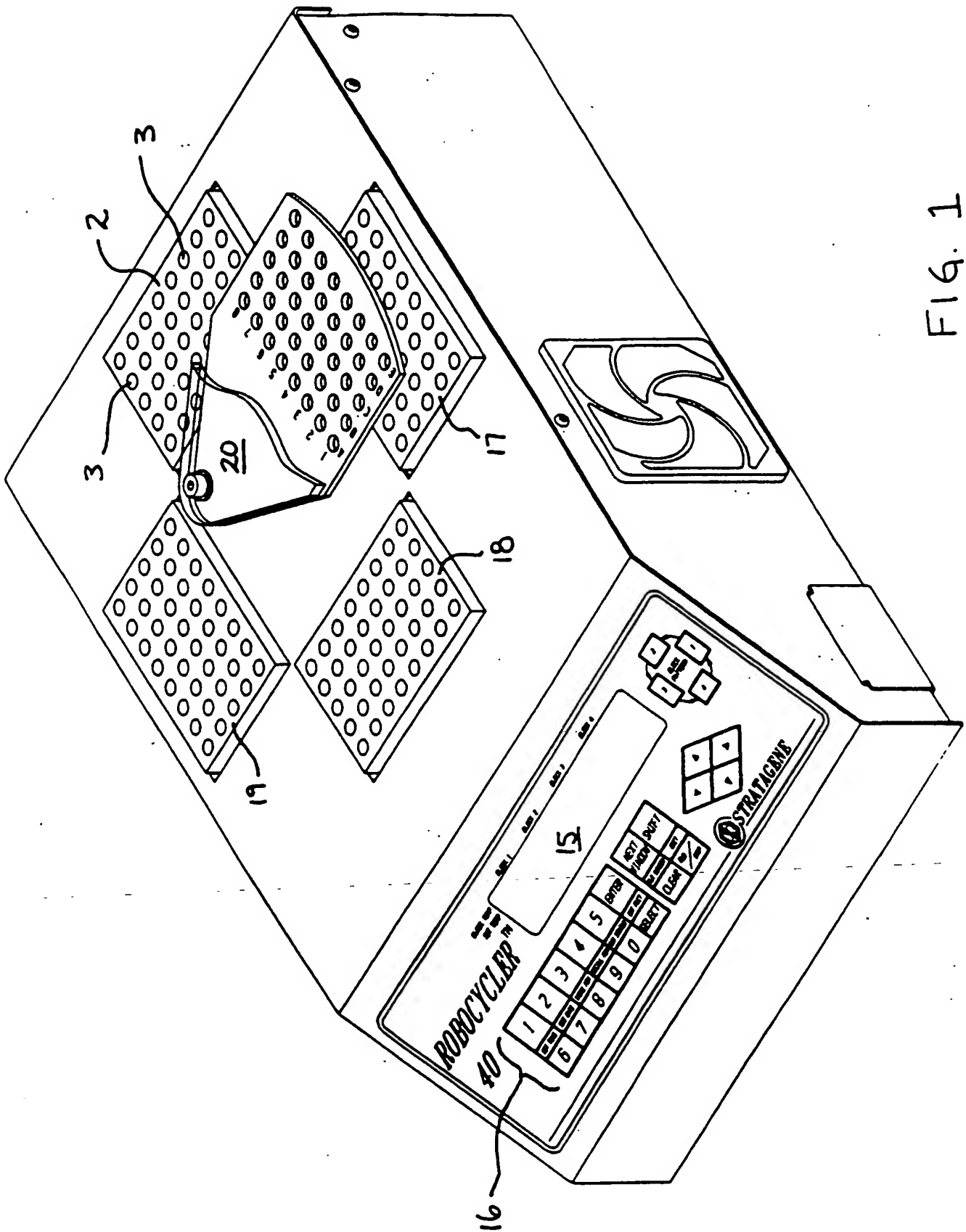
28. An apparatus for generating a temperature gradient across a heat conducting block according to claim 22 wherein said apparatus further comprises robot arm means, controlled by robot arm control means, for moving said reaction mixture 15 between said holding means.

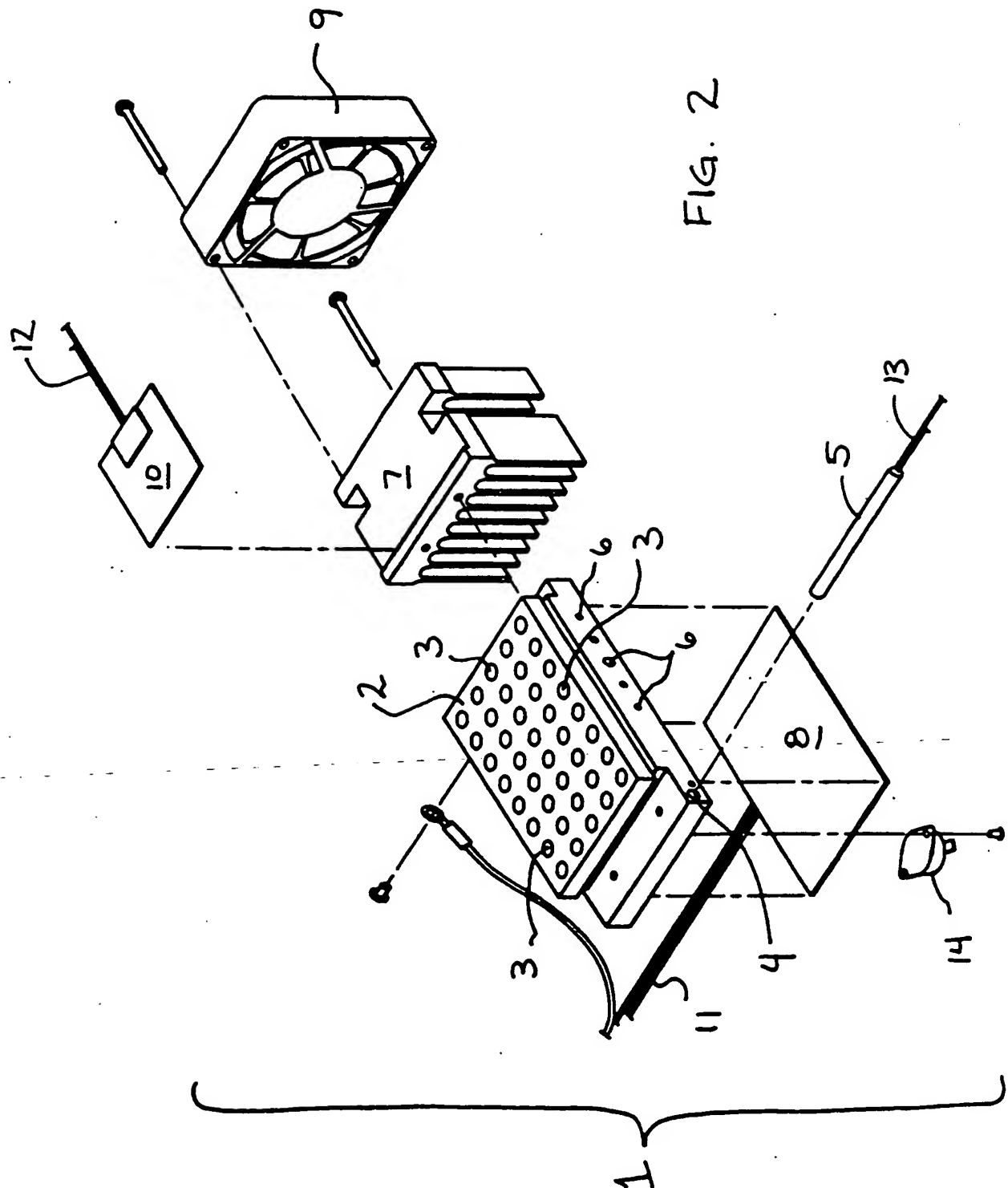
29. An apparatus for performing molecular biological reactions comprising at least one temperature controlled block, said block having a plurality of reaction mixture wells 20 spaced between first and second opposing portions and in an upper portion thereof, and a block heater positioned adjacent to said first opposing portion and capable of generating a temperature gradient between said first and second opposing portions.

25

30

35





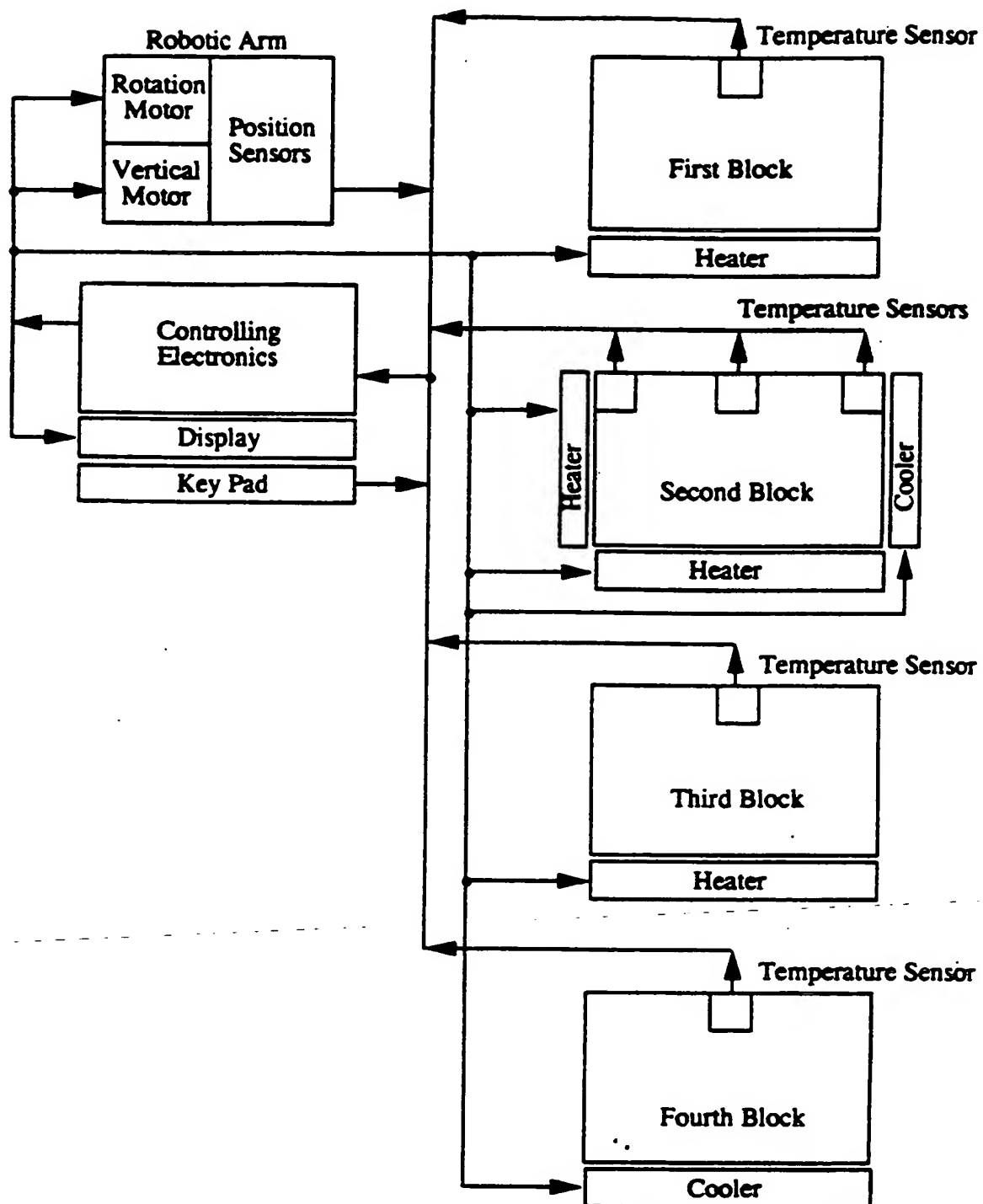


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12023**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12M 1/38

US CL : 165/14, 26, 30; 422/50, 68.1, 99, 104, 109; 435/290, 316, 809

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 165/14, 26, 30; 422/50, 68.1, 99, 104, 109; 435/290, 316, 809

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P -- Y	US, A, 5,255,976 (CONNELLY) 26 October 1993, see entire document.	1-4, 6, 8-14, 17-25, 27-29 ----- 5, 7, 15, 16, 26
X -- Y	US, A, 3,801,467, (NOBE et al.) 02 April 1974, see entire document.	1-4, 6, 8-14, 17- 25, 27-29 ----- 5, 7, 15, 16, 26
Y	TwinBlock Thermalcycler Advertisement, BioTech, Volume 14, No. 1, issued January 1993.	5, 15, 26

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

•	Special categories of cited documents:	•T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
•A	document defining the general state of the art which is not considered to be of particular relevance		
•E	earlier document published on or after the international filing date	•X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
•L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	•Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
•O	document referring to an oral disclosure, use, exhibition or other means		
•P	document published prior to the international filing date but later than the priority date claimed	•&	document member of the same patent family

Date of the actual completion of the international search 20 DECEMBER 1994	Date of mailing of the international search report FEB-03-1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAN M. LUDLOW <i>[Signature]</i> Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12023

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,981,801 (SUZUKI et al.) 01 January 1991, figures 7-8.	7, 16